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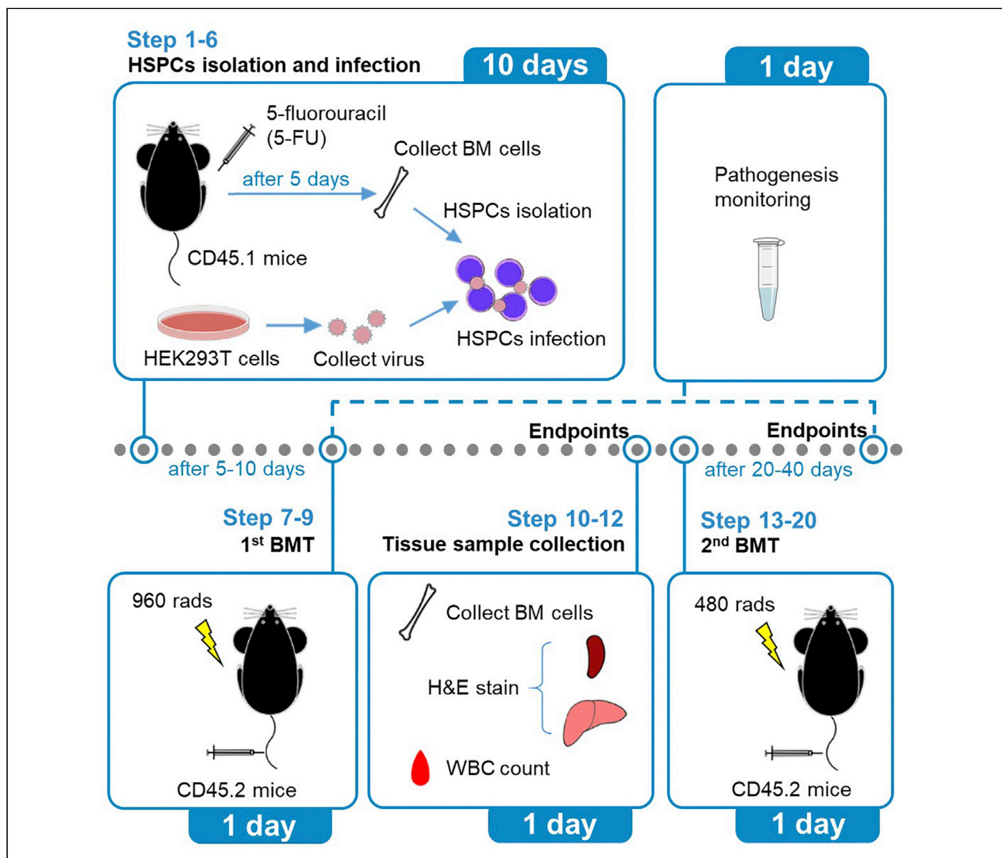
Protocol to establish a stable *MLL-AF9*_AML mouse model



Acute myeloid leukemia (AML) is one of the most common and fatal forms of hematopoietic malignancies. Here, we describe a mouse *MLL-AF9*_AML model to investigate AML. We have optimized the protocols for retrovirus infection, bone marrow transplantation (BMT), and leukemia monitoring to create a stable mouse model. In particular, we have used two rounds of BMT to enhance stability and efficiency. This model can be used to conduct drug administration and/or other interventions easily.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Optimized BMT accelerates full blast leukemia in recipient mice

Step by step details of two rounds of BMT and tissue sample collection

Protocols for monitoring AML pathogenesis after each BMT

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Protocol to establish a stable *MLL-AF9*_AML mouse model

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SUMMARY

Acute myeloid leukemia (AML) is one of the most common and fatal forms of hematopoietic malignancies. Here, we describe a mouse *MLL-AF9*_AML model to investigate AML. We have optimized the protocols for retrovirus infection, bone marrow transplantation (BMT), and leukemia monitoring to create a stable mouse model. In particular, we have used two rounds of BMT to enhance stability and efficiency. This model can be used to conduct drug administration and/or other interventions easily.

For complete details on the use and execution of this protocol, please refer to Zhao et al. (2022).

BEFORE YOU BEGIN

The steps below describe how to create an AML mouse model, i.e., the *MLL-AF9*_AML, for therapeutic investigation purposes. Stable *MLL-AF9* expression in primary donor cells is achieved through retroviral infection. Two times of BMT help accelerate the pathogenesis of full blast leukemia in recipient mice. This protocol also fits for the modeling of other AMLs that can be induced by single gene mutation or gene fusion, such as the *MLL-AF10*_AML, the *MLL-ENL*_AML, and the *AML-ETO9a*_AML, etc.

Preparation of animal

4-6 week-old B6.SJL (CD45.1) mice will be used as primary BMT donor mice, and should receive 150 mg/kg 5-Fluorouracil (5-FU) injection five days prior to the start retrovirus infection of HEK293T cells. All animals should be housed and maintained with a 12-h light/dark cycle at 23 ± 2°C.

> *Note:* The volume of injection is 40–50 μL per adult mouse, with the maximum not exceeding 100 μL.

Preparation of target plasmids

The MSCV-neo-*MLL-AF9* and retroviral package plasmid were kind gifts from Dr. Jianjun Chen of Beckman Research Institute of City of Hope.

Institutional permissions

All experiments should be carried out according to the ethical standards of animal care agencies. Randomization, allocation concealment and blind outcome assessment should be conducted

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throughout all the related experiments. Sample animals studied in this protocol were housed and maintained at the Laboratory Animal Center of Zhejiang University (Hangzhou, China), and experiments were carried out based on the approval of the animal care agency of Zhejiang University.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-Mo Ly-6G (Gr-1) Monoclonal Antibody (RB6-8C5), PE-Cyanine5 (1:100)	eBioscience	48-5931-82
CD11b Monoclonal Antibody (M1/70), Super Bright 600 (1:100)	eBioscience	63-0112-82
Anti-Mo CD45.1, PE-Cyanine5.5 (1:100)	eBioscience	45-0453-82
Chemicals, peptides, and recombinant proteins		
Recombinant mouse IL-3	PeproTech	213-13
Recombinant human IL-6	PeproTech	200-06
Recombinant mouse SCF	PeproTech	250-03
2-Mercaptoethanol (BME)	Macklin	M6230
5-Fluorouracil (5-FU)	Selleck	S1209
Geneticin sulfate (G418)	TargetMol	T6512
Lipofectamine™ 2000	Thermo Fisher Scientific	11668019
Opti-MEM	Gibco	31985062
RPMI-1640	Gibco	C11875500CP
DMEM (high glucose)	Gibco	C11995500BT
FBS	Gibco	10099141C
1 × PBS (pH7.4)	Solarbio	P1010
Penicillin-Streptomycin Liquid	Solarbio	P1400
BSA	Solarbio	A8020
HEPES	Solarbio	H8090
Sodium pyruvate	Sigma-Aldrich	P5280
Polybrene	Sigma-Aldrich	H9268
Ammonium chloride	Sinopharm	10009617
Potassium bicarbonate	Sinopharm	20030218
EDTA	Sinopharm	10009617
Anhydrous ethanol	Sinopharm	10009259
Human TruStain FcX™	BioLegend	422302
Staining buffer	BD Biosciences	554657
IC Fixation Buffer	eBioscience	00-8222-49
Critical commercial assays		
Lineage Cell Depletion Kit, mouse	Miltenyi Biotec	130-090-858
Experimental models: Cell liness		
HEK293T cells	ATCC	CRL-3216
Experimental models: Organisms/Strains		
B6.SJL (CD45.1) mouse	A gift from Dr. Fudi Wang	N/A
C57BL/6 mouse	Beijing Vital Laboratory Animal Technology	213
Recombinant DNA		
MSCV-neo-*MLL-AF9*	A gift from Dr. Jianjun Chen	N/A
pCL-ECO	A gift from Dr. Jianjun Chen	N/A
Software and Algorithms		
BD FACSDiva Software	BD Biosciences	https://www.bdbiosciences.com/zh-cn/products/software/instrument-software/bd-facsdiva-software
GraphPad Prism 8.0 software	GraphPad Software, Inc.	http://www.graphpad.com/scientificsoftware/prism/
Other		
60 mm dish	NEST	705001
6-Well plate	NEST	703001

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
15 mL centrifuge tube	NEST	601052
50 mL centrifuge tube	NEST	602052
1 mL Syringe (with 27G 0.5 inch Needle)	BD Biosciences	309623
3 mL Syringe (with 25G 1 inch Needle)	BD Biosciences	309582
0.22 μm filter	Millipore	SLGPR33RB
40 μm cell strainer	BD Falcon	352340
MS Columns	Miltenyi Biotec	130-042-201
0.5 mL MiniCollect tube K3E K3EDTA	Greiner Bio-One	450530
Cell counting plate	Countstar	12-0005-50
Sterile scalpel blade	JZ Surgical Instruments	J0B080 23#
X-ray irradiator	Rad Source	RS2000Pro
Refrigerated centrifuge	Thermo Fisher Scientific	ST16R
Countstar Automated Cell Counter	Countstar	IC 1000
Mouse tail vein injection device	KEW Basis	KW-XXY
Hematology analyzer	URIT, China	URIT-2900Vet Plus
Flow cell sorter	BD Biosciences	BD FACSAria III
OctoMACS Separator	Miltenyi Biotec	130-042-109
MACS MultiStand	Miltenyi Biotec	130-042-303
Lamp	OPPLE	E27

Note: Antibodies with other fluorescent labels can also be used. Other flow cytometry systems equipped with the requested laser channels would fit for the experimental purpose as well. Other cell counter equipment, e.g., the Countess (Thermo Fisher Scientific), can also be used.

MATERIALS AND EQUIPMENT

0.4% trypan blue solution		
Reagent	Final concentration	Amount
Trypan blue	0.4%	40 mg
1 × PBS	n/a	10 mL
Total	**n/a**	**10 mL**

Note: This solution should be used after filtering with 0.22 μm filter and can be stored at 24°C for long.

HEK293T medium		
Reagent	Final concentration	Amount
FBS	10%	1 mL
Penicillin-Streptomycin Liquid	1%	500 μL
DMEM (high glucose)	n/a	48.5 mL
Total	**n/a**	**50 mL**

Note: This medium can be stored at 4°C for 1 month.

BM washing medium		
Reagent	Final concentration	Amount
FBS	2%	1 mL
RPMI-1640	n/a	49 mL
Total	**n/a**	**50 mL**

Note: This medium can be stored at 4°C for 1 month.

ACK lysis buffer		
Reagent	Final concentration	Amount
NH_4Cl	155 mM	8.29 g
$KHCO_3$	10 mM	1.0 g
EDTA	0.1 mM	0.2 mL (500 mM)
Total	n/a	**1 L**

Note: This medium should be used after filtering with 0.22 μm filter and can be stored at 4°C for 1 month.

Progenitor cell culture medium		
Reagent	Final concentration	Amount
Recombinant mouse IL-3	10 ng/mL	100 ng
Recombinant human IL-6	10 ng/mL	100 ng
Recombinant mouse SCF	100 ng/mL	1 μg
BME	55 mM	0.55 mM
Penicillin-Streptomycin Liquid	1%	100 μL
HEPES	1%	100 μL
FBS	10%	1 mL
RPMI-1640	n/a	9 mL
Total	n/a	**10 mL**

Note: This medium should be made freshly on the day of use and kept storage at 4°C.

MCs buffer		
Reagent	Final concentration	Amount
BSA	0.5%	8.29 g
EDTA	2 mM	29.2 mg
1 × PBS	n/a	50 mL
Total	n/a	**50 mL**

Note: This buffer should be used after filtering with 0.22 μm filter and can be stored at 4°C for 1 month.

Frozen stock solution		
Reagent	Final concentration	Amount
FBS	20%	200 μL
DMSO	10%	100 μL
RPMI-1640	n/a	700 μL
Total	n/a	**1 mL**

Note: This solution should be made freshly on the day of use.

STEP-BY-STEP METHOD DETAILS

Retroviral particle production and mouse hematopoietic stem/progenitor cell (mHSPC) spinoculation

Timing: 10 day

The aim of this session is to produce retroviral particles encoding *MLL-AF9* oncogenic gene fusion, and infect mHSPCs isolated from CD45.1+ donor mice with this retrovirus.

1. Day 0: Seed HEK293T cells in 60 mm culture dishes at 0.5×10^6 cells per dish in 5 mL HEK293T medium, and culture the cells in 37°C, 5% CO_2 incubator.
   a. Cells are counted as follows: Add 20 μL cell suspension into a 1.5 mL tube. Mix well with 20 μL 0.4% trypan blue solution.
   b. Take out 20 μL of the mixture and add the mixture into the cell counting plate. Place the cell counting plate into the Countstar Automated cell counter and count the cell number.

   Note: 1×10^6 HEK293T cells will be needed for virus producing purpose for 1×10^6 bone marrow (BM) progenitor cells of one infection group.

2. Day 1: Transfect HEK293T cells.
   a. Change the medium of HEK293T cells with 4 mL fresh HEK293T medium before transfection.
   b. Prepare two 1.5 mL centrifuge tubes, label the tubes with Tube 1 and Tube 2, and then add 250 μL Opti-MEM per tube.
   c. Add plasmid and Lipofectamine™ 2000 into each tube according to the list below:

Transfection system		
NO.	Reagent	Amount
Tube 1	MSCV-neo-*MLL-AF9* plasmid	1.8 μg
	pCL-ECO plasmid	1.2 μg
Tube 2	Lipo2000	10 μL

   d. Mix each tube gently and incubate for 5 min at 24°C.
   e. Combine tube 2 with tube 1, mix slightly and incubate the mixture for 20 min at 24°C.
   f. Add the transfection mixture into HEK293T cells and culture the cells at a 37°C, 5% CO_2 incubator.

   Note: To obtain higher transfection efficiency, the ideal density of HEK293T cells is of 50%–60% confluence and well adherent.

3. Day 2: Insolate BM progenitor cells.
   a. Change medium of transfected HEK293T cells with 4 mL fresh HEK293T medium and 1.0 mM sodium pyruvate early in the morning.
   b. Euthanize donor mice. Disinfect the mice with 70% ethanol. Dissect the tibia and the femur of both rear arms, remove the muscles, adipose and disconnect the joints as much as possible (Figure 1).
   c. Put the bones into a 60 mm tissue dish containing 4 mL fresh BM washing medium. Flush BM cells into a new 60 mm tissue dish with 3 mL BM washing medium by use of a 3 mL syringe with 25 G 1 1/2 inch needle (Figures 2A and 2B).
   d. Eliminate cell debris and clumps by filtering the cell suspension with a 40 μm cell strainer, and collect the flow-through into a 50 mL centrifuge tube (Figures 2C and 2D). Wash the cell strainer with 1 mL BM washing medium.
   e. Spin down at 400 g for 8 min at 4°C.
   f. Aspirate the supernatant and resuspend the cell pellets with 20 mL ACK lysis buffer at 4°C for 8 min to remove the red blood cells lysis.
   g. Spin down at 400 g for 8 min at 4°C. Discard the supernatant and add 5 mL MCs buffer to re-suspend the cell pellets.
   h. Count cell numbers.

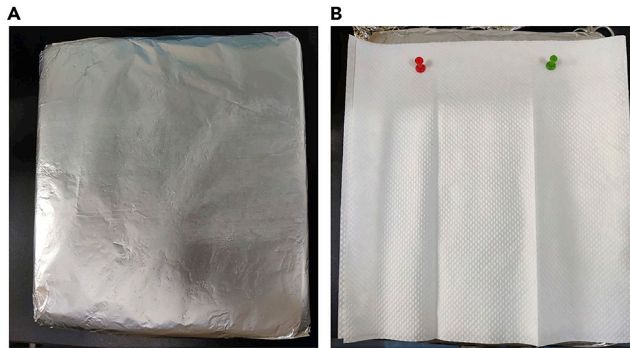

Figure 1. Preparation of an instant mouse fixation and operation board
(A) Wrap the foam board with aluminum foil.
(B) Cover the aluminum foil with 3 layers of paper towels. And then euthanized mouse can be fixed on top of the board for further operations.

i. Centrifuge cells at 400 g for 8 min. Aspirate the supernatant.
j. Use Lineage Cell Depletion Kit for magnetic labeling according to the manufacturer's protocol. Add 40 μL MCs buffer per 10^7 total cells to resuspend the cell pellets.
k. Add 10 μL Biotin-Antibody Cocktail per 10^7 cells and mix well. Incubate at 4°C for 10 min.
l. Add 30 μL MCs buffer and 20 μL Anti-Biotin MicroBeads per 10^7 total cells and mix well. Incubate at 4°C for 15 min.
m. Add 1 mL MCs buffer per 10^7 total cells to wash the cells. Spin down at 300 g for 10 min.
n. Aspirate the supernatant and add 500 μL MCs buffer per 5×10^7 total cells to resuspend the cell pellets.
o. Place the separation column in a MACS separator device. Rinse the separation column with 500 μL MCs buffer. Transfer the cell suspension to separation column and collect the pass-through.
p. Wash the separation column with 500 μL MCs buffer for three times.
q. Count the enriched progenitor cells. Centrifuge cells at 400 g for 8 min and discard the supernatant. Resuspend the cell pellets at a density of $1–5 \times 10^6$ cells/mL with progenitor cell culture medium. Culture the cells at 37°C, 5% CO_2 incubator.

Note: According to the instruction, the maximum volume of a MS column is 500 μL. According to our experiences, it is expected that one 5-FU injected healthy donor yields $2.5–3.5 \times 10^6$ BM cells. Other groups also reported the expected yields were around $3.04 \pm 0.79 \times 10^6$ four days after 150 mg/kg 5-FU treatment. (Shaikh et al., 2016).

4. Day 3: Infect BM progenitor cells.
   a. Pre-warm the centrifuge at 30°C.

⚠ CRITICAL: It is important to keep the temperature consistently at 30°C to ensure the transfection efficiency and cell survival.

   b. Collect the retrovirus and filter the virus medium with a 22 μm cell strainer into a 15 mL centrifuge tube. Change medium as the same as Day 2 for HEK293T cells.
   c. Add the 3.2 mL filtered retrovirus into one well of 6 well plates. Add 800 μL cell suspension into the virus soup.
   d. Add 4 μg/mL polybrene into the wells.
   e. Centrifuge cells at 550 g, 30°C for 3 h.
   f. Incubate the cells at 37°C, 5% CO_2 incubator for 1 h.
   g. Collect the BM cells into a 15 mL centrifuge tube and centrifuge at 400 g for 8 min.

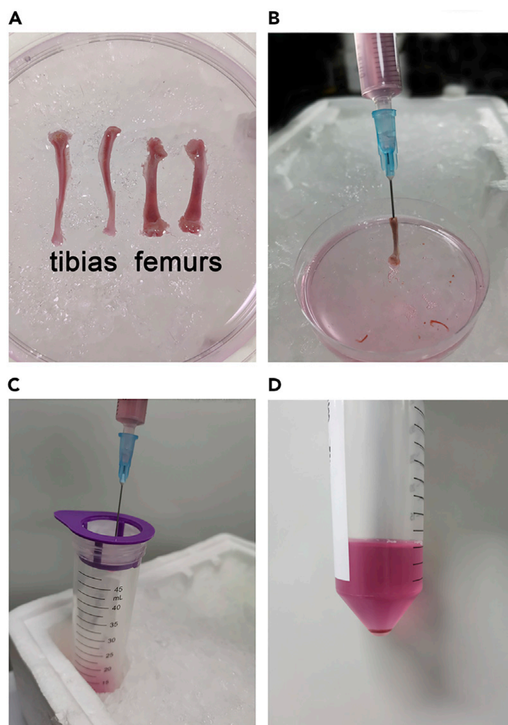

Figure 2. Harvest the bone marrow (BM) cells
(A) Dissect the tibias and femurs into a 60 mm tissue culture dish with 4 mL fresh BM washing buffer.
(B) Flush the BM cells.
(C and D) Filter the cell suspension through a 40 μm cell strainer.

   h. Aspirate the supernatant and resuspend the cell pellets with 4 mL progenitor cell culture medium.
5. Day 4: Repeat the procedure as Day 3 to infect BM progenitor cells again.
6. From Day 5–10: Add 50 mg/mL G418 into each well of the cell culture plate.

> ⚠ CRITICAL: Take care of progenitor cells; record cell numbers and viability every other day if necessary and keep cell density at 1–5× 10^6 cells/ml.

1st BMT

> ⏲ Timing: 1 day

In this session, transfected primary donor cells are transplanted into primary recipient mice to trigger primary AML.

7. Irradiate C57BL/6 (CD45.2) recipient mice at 960 rads in the morning.
8. Prepare donor cells for BM transplantation.
   a. Sacrifice one CD45.2 mouse (not irradiated) as helper and collect its BM cells according to the above procedure.
   b. Harvest transfected BM progenitor cells and filter the cell suspension with a 40 μm cell strainer into a 50 mL centrifuge tube to remove cell clumps.
   c. Count cell number and take out appropriate cells as BMT donor cells. Keep these cells in another 50 mL centrifuge tube.
   d. Add 1 × 10^6 helper cells per recipient mouse into collection tube containing donor cells.
   e. Centrifuge the cells mixture at 400 g for 8 min.
   f. Remove the supernatant and resuspend the cell pellets with 1 × PBS.
   g. Centrifuge cells at 400 g for 8 min.

h. Discard the supernatant. Add appropriate amount of 1 × PBS (100 μL per recipient mouse) into the cell pellets.

9. Transplant donor cells into recipient mice via tail vein injection.
   a. Pre-warm the recipient mice adequately with a warm lamp, for about 10–15 min, to dilate blood vessels prior to injection.
   b. Use a 1 mL syringe and a 25 G 1 inch needle to mix the cell.
   c. Change the needle with a 27 G 0.5 inch one and inject 100 μL of the cell suspension per mouse through tail veins.
   d. Feed recipient mice with antibiotic-containing water.

Note: Pay attention to the health condition of the irradiated mice. If necessary, give some auxiliary care, e.g., water gel, soft food, etc., in the first one or two weeks after BMT.

Tissue sample collection from leukemia mice

Timing: 1 day

In this session, BM cells from the primary leukemic recipient mice are collected.

10. Harvest the BM cells and spleen according to the procedure 3b-i when the recipient mice are meeting the endpoints.

Optional: Harvest the blood, liver and spleen according to your experimental purposes (Figure 3).

11. Prepare 0.1–0.2 × 10^6 1st BMT recipient mice' BM cells as donor cells for each 2nd recipient mouse in 2nd BMT.

Note: Since engraftment (i.e., CD45.1+ population) of 1st BMT recipients at the end point is usually above 85% (Figure 4), these BM cells (i.e., 2nd BMT donor cells) are considered to be leukemic cells with malignant expansion, and can be used as 2nd BMT donor cells directly. If the primary recipients' final engraftment is below 85%, purify the leukemic cells through flow cytometry isolation (see step 20 for details).

12. Freeze remaining cells in liquid nitrogen with frozen stock solution.

Pause point: Cells can be stored in liquid nitrogen for several months.

2nd BMT

Timing: 20–40 days

In this session, AML cells collected from the primary leukemic recipients are transplanted as donor cells into secondary recipient mice to trigger 2nd leukemia.

13. Collect the BM cells from primary BMT leukemic mice according to the procedure shown above when the mice reach the endpoints. Freeze 5 × 10^6 cells per tube in liquid nitrogen with frozen stock solution.
14. Thaw one vial of primary BMT leukemic mice BM cells one day before 2nd transplantation as the 2nd BMT donor cells.
15. Irradiate C57BL/6 (CD45.2) mice as 2nd BMT recipients at 480 rads in the morning.
16. Prepare 2nd BMT donor cells (0.1–0.2 × 10^6 cells per mouse) according to the procedure 8 b-c and e-h.

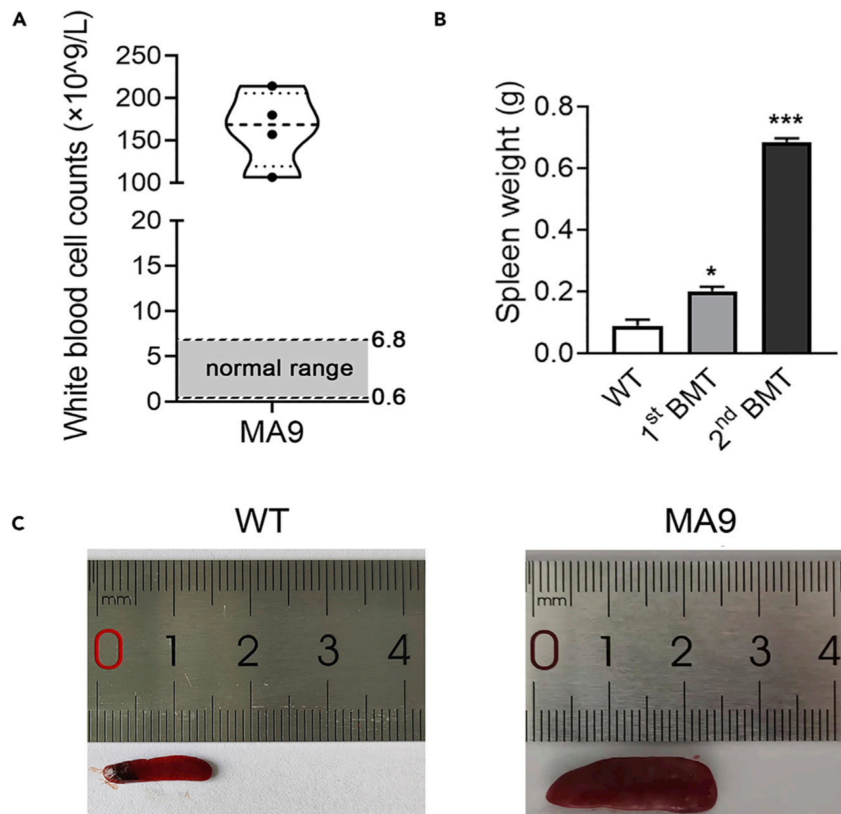

Figure 3. WBC counts and spleen weights of non-treated C57BL/6 mice, and leukemic recipient mice after 1st BMT or 2nd BMT

(A) WBC counts of *MLL-AF9*_AML mice at the endpoints of 2nd BMT.

(B) Spleen weights of non-treated C57BL/6 mice, and leukemic recipient mice after 1st BMT or 2nd BMT.

(C) Spleen images of a non-treated C57BL/6 mouse and a *MLL-AF9*_AML mouse. Mean ± SEM, ***$p<0.001$, two-tailed t-test.

17. Inject donor cells into tail veins according to the procedure 9.
18. Monitor the engraftment of donor cells in recipient mice, and monitor leukemia pathogenesis by collecting peripheral blood (PB) and measuring the percentage of CD45.1+ cell via flow cytometry.
    a. Restrict a mouse manually or using a mouse restainer for blood collection.
    b. Aseptically prepare the tail with 70% ethanol.
    c. Nick the tail with a sterile scalpel blade on a lateral side.
    d. Collect 50 μL blood in a 0.5 mL MiniCollect tube.
    e. Press the tail to stop bleeding and return the mouse into the cage.
    f. Use 1 mL ACK lysis buffer to lyse the red blood cells and move the lysis in to a new 1.5 mL tube. Incubate at 4°C for 8 min.
    g. Spin down at 400 g for 8 min at 4°C and remove the lysis.
    h. Use 1 mL ACK lysis buffer to resuspend cell pellets. Repeat the incubation and centrifugation.
    i. Use 1 mL ice-cold 1 × PBS to resuspend cell pellets and spin down at 400 g for 8 min at 4°C. Remove the supernatant.
    j. Sample preparation and detection for flow cytometry according to the steps 20b–g.

Pause point: Fixed flow cytometry samples can be stored in 4°C for 2–3 days before testing.

Optional: Drug administration or other manipulation can be carried out in this period.

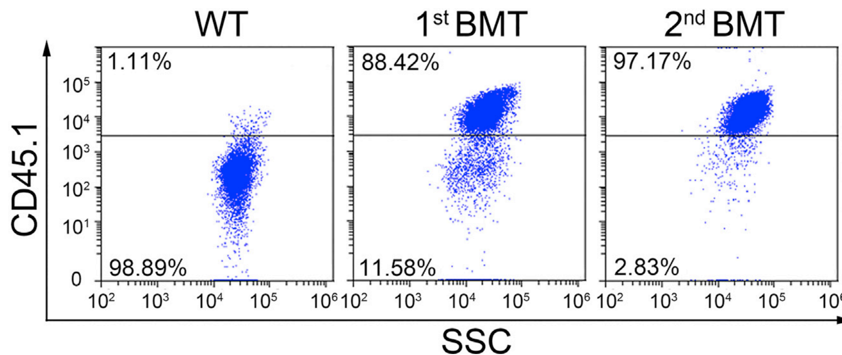

Figure 4. Flow analysis showing the engraftment of CD45.1+ cells in BM of non-treated C57BL/6 mice, 1st BMT or 2nd BMT recipient mice at the endpoints

Pathological sample collection and pathogenesis monitoring

Timing: 1 day

In this session, AML pathogenesis after each BMT is monitored through flow cytometry analysis.

19. Collect PB from heart into 0.5 mL MiniCollect tube by a 1 mL syringe with 27 G 0.5 inch needle and test WBC (Figure 3A). Harvest the BM cells and weigh the liver and spleen of leukemia mice (Figures 3B and 3C).

Optional: Collect liver and spleen tissues, perform paraffin embedding, slide cutting and H&E staining, to investigate leukemic cell infiltration condition. Observe cell morphology with cytospin specimens of BM cells.

20. Detecting leukemic cell engraftment and differentiation through BM cells and flow cytometry analysis.
    a. Collect 1×10^6 leukemia mice BM cells and wash with ice cold 1 × PBS.
    b. Add 30 μL BD staining buffer and 0.3 μL Human TruStain FcX™ to resuspend the cell pellets.
    c. Blend the cells by flicking and stay at 24°C for 10 min.
    d. For the leukemic cell engraftment test, add 0.5 μL anti-mouse CD45.1- PE-Cyanine5.5 into TruStain FcX™ blocking cells. Stain cell samples with anti-mouse CD11b-Super Bright 600 and anti-mouse Gr-1- PE-Cyanine5 antibodies for cell differentiation test. Keep a blank control without adding any antibodies or simple IgG.
    e. Incubate at 4°C for 25 min. Avoid light.
    f. Add 1 mL ice cold 1 × PBS to wash away excess antibodies. Centrifuge cells at 400 g for 8 min.
    g. Discard the supernatant and resuspend the cell pellets with 400 μL IC Fixation Buffer and then load for flow cytometry analysis on BD FACSAria III.

Pause point: Fixed flow cytometry samples can be stored in 4°C for 2–3 days before testing.

EXPECTED OUTCOMES

This protocol describes a mouse model of acute myeloid leukemia for therapeutic investigation purposes. Drug administrations or other inventions can be conducted when the engraftments reach 15%–20%, i.e., the onset of leukemia. The engraftment can be tested by collecting PB and measuring the percentage of CD45.1+ cell via flow cytometry every 10 days post BMT. Usually it takes 60–90 days for the primary BMT recipient mice and 25–40 days for the secondary BMT

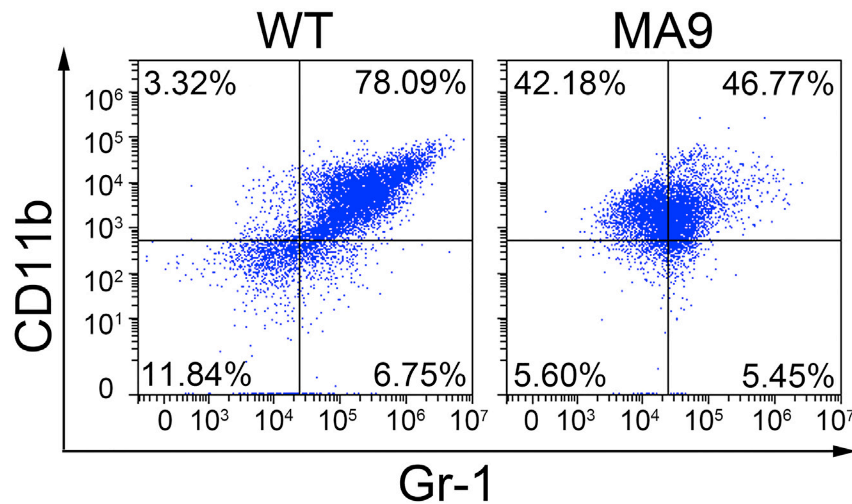

Figure 5. Flow analysis showing cell lineages of BM cells of *MLL-AF9*_AML mice and C57BL/6 controls

recipient mice to develop full blast leukemia. Significant differences can be found in spleen size between non-treated C57BL/6 mice, 1st BMT leukemic recipients and 2nd BMT leukemic recipients, indicating the infiltration of AML cells into spleen of the recipient mice (Figure 3). The engraftment of *MLL-AF9* leukemic cells (CD45.1+) can be observed through flow cytometry analysis in 1st BMT leukemic recipients and 2nd BMT leukemic recipients (Figure 4). The proportion of $Gr1^+/CD11b^+$ cells was remarkably reduced in BM cells of *MLL-AF9* leukemic recipient mice as compared with BM cells of non-treated C57BL/6 mice, indicating the increase of immature neutrophilic cell proportion in AML recipient mice (Figure 5).

LIMITATIONS

This protocol provides a complete method to establish the *MLL-AF9*_AML disease model through two rounds of BMTs (Jiang et al., 2012, 2016, 2017). Because of the lethal dosage of irradiation, especially in the primary BMTs, it is inevitable that a small proportion (around 10–20%) of the recipient mice might turn weak or even die after exposure. The other limitation is that it takes relatively long for the primary BMT recipients to develop leukemia. For example, it takes up to 9 months for the *AML-ETO9a* primary recipients to reach the onset of leukemia.

TROUBLESHOOTING

Problem 1

The yield of HSPCs is not enough (step 3 of step-by-step method details).

Potential solution

After injecting with 150 mg/kg 5-FU, the 6-week-old mouse should yield at least 3.5×10^6 BM cells. To avoid low yield of HSPCs, we suggest: (1) Make sure that the dose of 5-FU is 150 mg/kg per adult mouse of body weight; (2) Ensure that all BM cell collection processes are carried out on ice, and shorten the whole collection procedure as much as possible; (3) The capacity of MS column is 1×10^7 magnetically labeled cells from up to 2×10^8 total cells according to the manufacturer's protocol. An excessive count of total cells could clog the column, so that the cell fluid won't flow through smoothly. For this issue, we recommend to use more than one MS column and combine the pass-through, or use a LS column, whose capacity is 10 times larger than the MS column.

Problem 2

The virus titer is not high enough (step 5 of step-by-step method details).

Potential solution

Given the extremely high expansion capability of *MLL-AF9*-AML cells, the workable virus titer range is quite broad. In order to obtain optimal retrovirus production, make sure the HEK293T cells are in good condition. All the plasmids used for transfection should be of proper purity and concentration, and endotoxin free.

Problem 3

Low cell viability of HSPCs after spinoculation and G418 selection (step 9 of step-by-step method details).

Potential solution

The centrifuge should be preheated before spinoculation, and ensure the temperature stay stable during the 3 h' spinning. It is not recommended to change culture medium immediately after spinoculation. The infected HSPCs should be incubated at a 37°C, 5% CO_2 incubator for 1 h before changing medium. Cell viability should be monitored and cell density should be adjusted from time to time after two times of spinoculation as well as the G418 selection period.

Problem 4

The recipient mice die after irradiation and BMT (steps 10–12 of step-by-step method details).

Potential solution

It is highly recommended to give supportive care to the recipient mice in the first one or two weeks after irradiation and BMTs. Antibiotics can be added into drinking water to prevent infection. Soft food and water gel can be used. Donor cells for BMT should be sieved with a 40 μm cell strainer to remove large cell clumps prior to cell counting, and thus to prevent blood vessels from being clogged after tail vein injection. All the surgical instrument should be sterilized at 103.4k Pa steam pressure (121.3°C) for 20 min in advance. The injection process should be quick and skilled, and bleeding should be stopped in time after surgery.

Problem 5

The engraftment of transplanted cells is low or slow (steps 19 and 20 of step-by-step method details).

Potential solution

The BM CD45.1+ cell proportion of a primary AML recipient mouse is usually above 85%, and these cells are with high malignant expansion potency. Obvious engraftment (i.e., CD45.1+>10%) of these donor cells can be often detected 20 days post 1st BMT or 10 days post 2nd BMT through tail vein blood collection and flow cytometry analysis, as shown in steps 19 and 20. The speed of engraftment is closely related with the malignant potency of the donor cells, as well as the condition of the recipient mice. It is recommended to use cells with high viability and malignant expansion potency as donor cells. MTT assays can be done prior to BMT for the purpose of selecting optimal donor cells. Different kinds of recipient mice have slightly different responses to irradiation. The irradiation dose can be adjusted among 800–960 rads for 1st BMT, and 400–500 rads for 2nd BMT, with one dosage or two split dosages. In some cases, BM suppressive drugs, e.g., Busulfan, can be an alternative choice.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Xi Jiang (xjiang@zju.edu.cn).

Materials availability

No new reagent or materials were generated in this study.

Data and code availability

No new datasets or code was generated in this study.

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AUTHOR CONTRIBUTIONS

J.L., H.Z., L.Y., and X.J. analyzed the data and wrote the paper.

DECLARATION OF INTERESTS

The authors declare no conflicts of interest.

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